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Elevated CSF N-acetylaspartylglutamate suggests specific molecular diagnostic abnormalities in patients with white matter diseases

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ABSTRACT

Background: In order to identify biomarkers useful for the diagnosis of genetic white matter disorders we compared the metabolic profile of patients with leukodystrophies with a hypomyelinating or a non-hypomyelinating MRI pattern. Methods: We used a non-a priori method of *in vitro* ^{1}H-NMR spectroscopy on CSF samples of 74 patients with leukodystrophies. Results: We found an elevation of CSF N-acetylaspartylglutamate (NAAG) in patients with Pelizaeus–Merzbacher disease (PMD)—*PLP1* gene, Pelizaeus–Merzbacher-like disease—*GJC2* gene and Canavan disease—*ASPA* gene. In the PMD group, NAAG was significantly elevated in the CSF of all patients with *PLP1* duplication (19/19) but was strictly normal in 6 out of 7 patients with *PLP1* point mutations. Additionally, we previously reported increased CSF NAAG in patients with *SLC17A5* mutations. Conclusions: Elevated CSF NAAG is a biomarker that suggests specific molecular diagnostic abnormalities in patients with white matter diseases. Our findings also point to unique pathological functions of the overexpressed PLP in PMD patients with duplication of this gene.

1. Introduction

Elevation of CSF N-acetylaspartylglutamate (NAAG) in some leukodystrophies has been previously described but its relationship to disease is poorly understood, with the exception of Canavan disease where the elevation of N-acetylaspartate (NAA) may be directly implicated [1].

Using two capillary electrophoresis systems, NAAG has been shown to be elevated in the CSF of 29 out of 32 patients with a clinical presentation of Pelizaeus–Merzbacher disease (PMD) [2]. This included 22 patients with *PLP1* mutations and 7 patients with Pelizaeus–Merzbacher-like disease (PMLD). However, the nature of the mutations was not reported either in the PMD or the PMLD groups. An increase of NAAG was also observed in the CSF of one patient with Canavan disease [3], one PMLD patient harboring a homozygous deletion in the *GJC2* gene [4] and two patients with a severe hypomyelination pattern of unknown cause [5]. In addition, we recently reported that CSF NAAG was increased in 6 patients with sialic acid storage disease (SASD) associated with *SLC17A5* mutations [6].

Therefore, we decided to use proton NMR spectroscopy (^{1}H-NMRS) to determine whether the levels of CSF NAAG can facilitate the molecular diagnosis of patients with leukodystrophies with a hypomyelinating or a non-hypomyelinating pattern on brain MRI [7].

2. Material and methods

2.1. Patients

Seventy-four children and adults from two referral centers for neurogenetics and neurometabolism were enrolled in clinical protocols approved by the ethics committees of the Hôpital de Clermont-Ferrand, France, and the National Institutes of Neurological Disorders and Stroke, Bethesda, MD, USA. Written informed consent was obtained from all patients or their legal guardians.

The cohort of patients with leukodystrophies with a hypomyelinating pattern consisted of (i) 26 male patients with *PLP1* mutations presenting as PMD or X-linked spastic paraplegia type 2 [8]–PMD/SPG2 patients–including 19 patients with *PLP1* duplication and 7 patients with *PLP1* missense or splice mutations (Table 1); (ii) 9 patients with PMLD

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including one patient with GJC2 mutations; and (iii) 10 patients with a leukodystrophy of unknown etiology despite extensive metabolic and genetic testing. The rest of the cohort consisted of 14 patients with leukodystrophies with a non-hypomyelinating pattern–including patients with Canavan disease, merosin deficiency, MLC1 mutation and complex I deficiency–and 15 diseased controls with complex neurological diseases but normal brain MRI.

2.2. Methods

In order to assess clinical severity, we used a score previously validated in patients with *PLP1* mutations [9]. Briefly, form 0 referred to absence of motor achievement; form 1–head control between 2 and 4 years; form 2–sitting position between 2 and 5 years; form 3–sitting position between 1 and 2 years, walk with support at a mean age of 3.5 ± 1.5 years (range 2–6); form 4–autonomous walking.

Frozen CSF–stored at −80 °C–were prepared for ^{1}H-NMRS with minimal handling [5,10]. First, CSF samples were deproteinized using a 10 kDa filter (Nanosep, Omega) to avoid interference from high molecular weight species such as lipoproteins. Before use, the filter was washed twice with water by centrifugation to remove glycerol. A 100 µl aliquot of 3.89 mM [trimethylsilyl]-2,2,3,3-tetradeuteropropionic acid in ^{2}H2O (TSP-^{2}H2O, Aldrich) was then added to 500 µl of the ultrafiltrate, providing a chemical shift reference ($\delta = 0.00$ ppm), a concentration reference and a deuterium lock signal. The pH of the ultrafiltrate was adjusted to 2.50 ± 0.05 with concentrated HCl. Finally, 500 µl of the sample was placed in a 5 mm NMR tube (Wilmad Royal Imperial). The proton NMR spectra were determined on an Avance-500 SB spectrometer (Bruker, France) equipped with a 5 mm BBI (broad-band inverse) probe; samples were not spun. Spectra were collected at 25 °C and consisted in 32 K data points with a spectral width of 6000 Hz and a total acquisition times of 27 min. A 90° radiofrequency pulse, following a water signal presaturation of 10 s, was used for each 128 scans. Shimming of the sample was performed on the deuterium signal until the resonance line width for TSP was <1 Hz. Before a Fourier transformation into 64 K data points, a sine window multiplication (sine bell shift of 90°) was used to reduce noise. The phase and the baseline were corrected manually using the spectrometer software (X-Win NMR 3.5, Bruker, France). Two-dimensional proton NMRS, ^{1}H–^{1}H correlation spectroscopy (COSY), was performed to confirm the identification of metabolites of interest in patients' body fluids. The spectra were recorded at 500 MHz using 4 k datapoints in *F2* and a spectral width of 6002 Hz. 256 Increments and 16 scans per increment were used. The *TR* was 1.5 s, during which the water resonance was presaturated. Prior to Fourier transformation, a sine window multiplication was applied in both time domains. The values of NAAG were calculated by comparison with the known concentration of the internal standard (TSP).

A one-way between groups analysis of covariance was conducted to compare the levels of CSF NAAG between PMD/SPG2 patients with *PLP1* duplication and *PLP1* point mutations using age and clinical score as covariates. Preliminary checks were conducted to ensure that there was no violation of the assumptions of normality, linearity, homogeneity of variances and homogeneity of regression slopes.

Table 1
Genetic, biochemical and clinical characteristics of PMD/SPG2 patients.

PLP1 mutation	NAAG (µmol/L)[a]	Age	Clinical score
p.Cys109TrpfsX11	ND	3	3
p.Asp103ThrfsX12	ND	17	3
p.Val219Phe	ND	38	3
c.IVS4 + 1G>A / p.Val209GlufsX27	ND	9	3
p.Leu223Phe	38	10	0
p.Cys220Tyr	ND	10	3
p.Asp103ThrfsX12	ND	18	4
Duplication	30	2	1
Duplication	36	6	3
Duplication	40	2	2
Duplication	65	6	3
Duplication	89	5	3
Duplication	32	17	2
Duplication	84	8	2
Duplication	60	11	3
Duplication	47	4	3
Duplication	106	13	2
Duplication	36	8	3
Duplication	56	5	2
Duplication	205	8	1
Duplication	88	9	1
Duplication	55	3	1
Duplication	32	5	2
Duplication	23	3	1
Duplication	53	5	3
Duplication	52	7	2

[a] Diseased controls, mean = 5 ± 1 µmol/L (n = 15). ND: below detection limit (<3 µmol/L).

3. Results

3.1. Elevated CSF NAAG in patients with PLP1, GJC2 and ASPA mutations

In a 1-dimensional ^{1}H-NMR spectrum (pH = 2.5), NAAG presents as a prominent singlet resonance at 2.04 ppm and smaller resonances at 2.01 and 2.23 (multiplets), 2.47 (triplet), 2.81 and 2.91 (doublet of doublets), 4.32 (doublet of doublets), and 4.72 ppm (doublet of doublets) [11]. Characteristic cross peaks in 2-dimensional COSY ^{1}H-NMRS are shown in Fig. 1.

Compared to our group of disease controls, *in vitro* ^{1}H-NMRS of CSF showed a significant elevation of NAAG in the CSF of PMD/SPG2 patients compared to controls (47±44 µmol/L vs. 5±1 µmol/L, $p<0.001$). The mean values of NAAG were normal in (i) patients with PMLD, (ii) patients with other leukodystrophies with a hypomyelinating MRI pattern, and (iii) patients with leukodystrophies with a non-hypomyelinating pattern (Fig. 2). However, ^{1}H-NMRS revealed a mild to moderate elevation in the CSF of one PMLD patient associated with *GJC2* compound heterozygous mutations (14 µmol/L), one patient with Canavan disease associated with *ASPA* mutations (17 µmol/L), and two patients presenting with a hypomyelinating leukodystrophy of unknown etiology (33 and 60 µmol/L respectively) (Fig. 2). Of note, we did not observe any CSF elevation of NAA except in the CSF of the patient with Canavan disease (98 µmol/L, Fig. 3).

3.2. Association of elevated CSF NAAG with the nature of the PLP1 mutation rather than clinical severity

^{1}H-NMRS revealed an elevation of NAAG in the CSF of all 19 patients with *PLP1* duplication (Table 1 and Fig. 2). Conversely, CSF NAAG was strictly normal in 6 out of 7 patients with *PLP1* missense, nonsense or splice mutations (Table 1 and Fig. 2). The only patient with *PLP1* mutations who displayed a significant elevation of CSF NAAG presented with the most severe clinical presentation—clinical score of 0 (Table 1). However, there was no correlation between the levels of CSF NAAG and clinical severity among the cohort of PMD/SPG2 patients, or among patients with *PLP1* duplication only. There was no correlation either between CSF NAAG and age. Moreover, when adjusting for both age and clinical severity, we found a significant elevation of NAAG in patients with *PLP1* duplication versus *PLP1* point mutations ($p = 0.012$, partial eta squared = 0.265). Therefore, the elevation of NAAG in the CSF of PMD/SPG2 patients strongly suggests the nature of the mutation–i.e. duplication in the *PLP1* gene–rather than clinical severity.

4. Discussion

In this study, using *in vitro* ^{1}H-NMRS we showed that NAAG is always elevated in the CSF of patients with *PLP1* duplication. We also confirmed

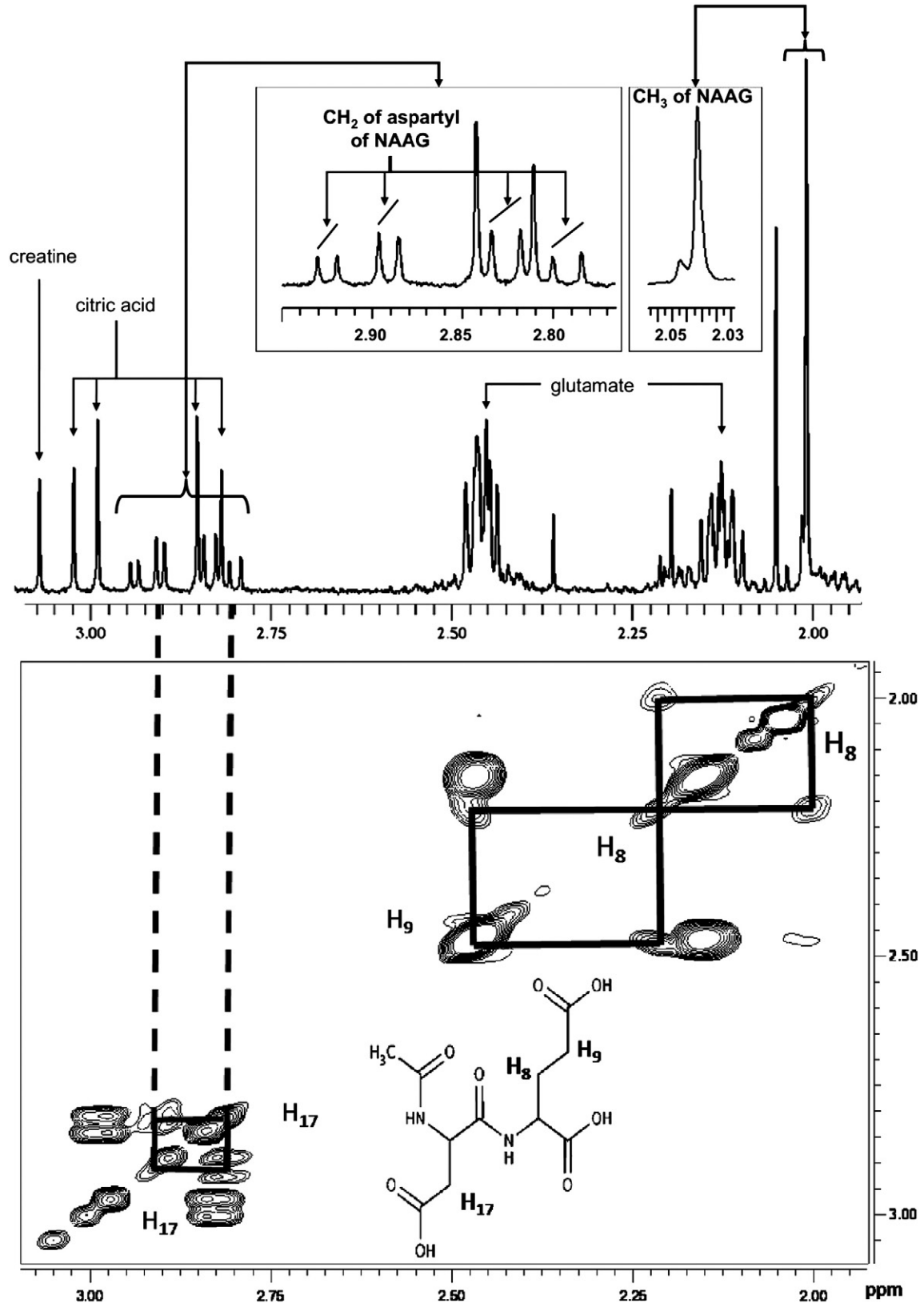

Fig. 1. One-dimensional and 2-dimensional (COSY) ^{1}H-NMR spectra of NAAG in CSF. The upper part shows the methyl signal (CH_3 of NAAG) in the 1D spectrum (2.04 ppm). The lower part shows the correlations between the two diastereotopic protons of CH_2 (H17)–2 doublets of doublets at 2.91 and 2.85 ppm–and the correlations between the two diastereotopic protons of CH_2 (H8)–multiplets at 1.99 and 2.23 ppm–and the two protons of CH_2 (H9)–triplet at 2.43 ppm–in the 2D spectrum. The signals of protons of CH_2 (H8) and CH_2 (H9) are not visible on the 1D spectrum because of their small intensities and overlap with glutamate and methyl signals.

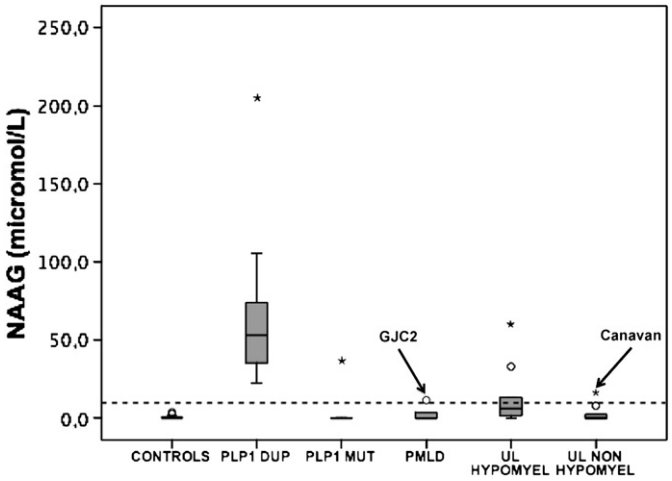

Fig. 2. Values of CSF NAAG in the cohort of 74 patients. The dotted line represents the upper limit of normal values of CSF NAAG (12 μmol/L [5]). DUP: duplication; MUT: point mutation; PMLD: Pelizaeus–Merzbacher-like disease; UL: unknown leukodystrophy. Circles and stars denote outliers that are farther than 1.5 and 3 interquartile ranges respectively from the nearer edge of the box.

increased CSF NAAG in patients with *GJC2* mutations and Canavan disease [2,3]. Despite the elevation of CSF NAAG in one patient with a *PLP1* point mutation displaying a very severe form of the disease, we did not find any correlation between the levels of CSF NAAG and clinical severity in PMD/SPG2 patients, which is consistent with our recent findings in SASD patients [6]. Since we had relatively few patients with mutated *PLP1* and severe clinical score (0–1), we cannot completely exclude that some patients with severe missense mutation also have elevated CSF NAAG. Nevertheless, there was a marked difference in the levels of CSF NAAG between the patients with *PLP1* duplication and those with *PLP1* point mutations, even when adjusting for clinical severity. Therefore, we suggest that quantifying NAAG levels in CSF of patients with white matter diseases can be a useful and simple tool to orient molecular diagnostic testing, especially in hypomyelinating leukodystrophies. Of note, *in vivo* ^{1}H-MRI spectroscopy is unable to provide similar information since the technique cannot discriminate between the NAA and NAAG peaks unlike *in vitro* ^{1}H-NMRS (Fig. 3). Consequently, the relatively high NAA concentrations in brain tissues prevent the measurement of NAAG *in vivo*. The detection limit of ^{1}H-NMRS for CSF NAAG is higher than of techniques such as two capillary electrophoresis [2] or mass spectrometry (personal observation). However, contrary to these techniques, ^{1}H-NMRS requires no derivatization or extraction. It can therefore simultaneously detect compounds of different nature which allows the non-a priori identification of new metabolic abnormalities in patients with uncharacterized or known genetic diseases [6,12].

The absence of correlation between CSF NAAG and clinical severity in PMD/SPG2 and SASD patients, as well as the observed neuroprotective effect of increased extracellular NAAG during GCPII inhibition [13], suggest that NAAG is an unlikely contributor to the hypomyelination phenotype observed in these patients. NAAG undergoes a tricellular metabolic sequence wherein it is synthesized in neurons from N-acetylaspartate (NAA) and L-glutamate, hydrolyzed to NAA and glutamate by astrocytes and further hydrolyzed to L-aspartate and acetate by oligodendrocytes [14]. NAA was also shown to provide acetyl groups for myelin synthesis [15]. It is therefore possible that the elevation of CSF NAAG in some hypomyelinating disorders reflects a compensatory mechanism for the altered maturation of oligodendrocytes in an effort to enhance myelin synthesis.

The elevation of CSF NAAG in patients with *PLP1* duplication, but usually not in patients with point mutations, favors a specific effect of the over-expressed wild-type *PLP1* gene as supported by *in vitro* and preclinical *in vivo* studies [16,17]. Overexpression of wild-type PLP in the CNS may affect the integrity of neurons as suggested by the increased mortality of neurons when cultured in the presence of conditioned media from PLP overexpressing cells but not from DM20–a splice variant of PLP–overexpressing cells. This effect of conditioned media may be mediated by a negative pH shift elicited by PLP [18] and could be related to the similarity of PLP and its four hydrophobic domain motif to proteins that are known to function as pores or channels [19]. Interestingly, abnormal connexin 47 associated with *GJC2* gene mutations was reported to alter the function of intercellular channels [20]. The sialin transporter, which was recently found to also function as an aspartate transporter [21], may have a role in neuronal secretory processes due to its additional non-lysosomal localization [22]. Therefore, the implication of PLP1, connexin 47 and sialin in the formation of ion channels/transporters in membranes suggests increased excretion of NAAG into the extracellular compartment. The lack of concurrent elevation of NAA further supports the hypothesis that NAAG elevation in CSF of patients with *PLP1* duplication results from excessive neuronal release of NAAG rather than from its over-production. We found two patients presenting with hypomyelination on brain MRI and increased CSF NAAG but without mutations of *PLP1*, *GJC2* or *SLC17A5*. A similar entity has been previously reported [5]. In these cases, a candidate genes approach should focus on genes encoding for proteins related to the structure and function of ion channels or transporters.

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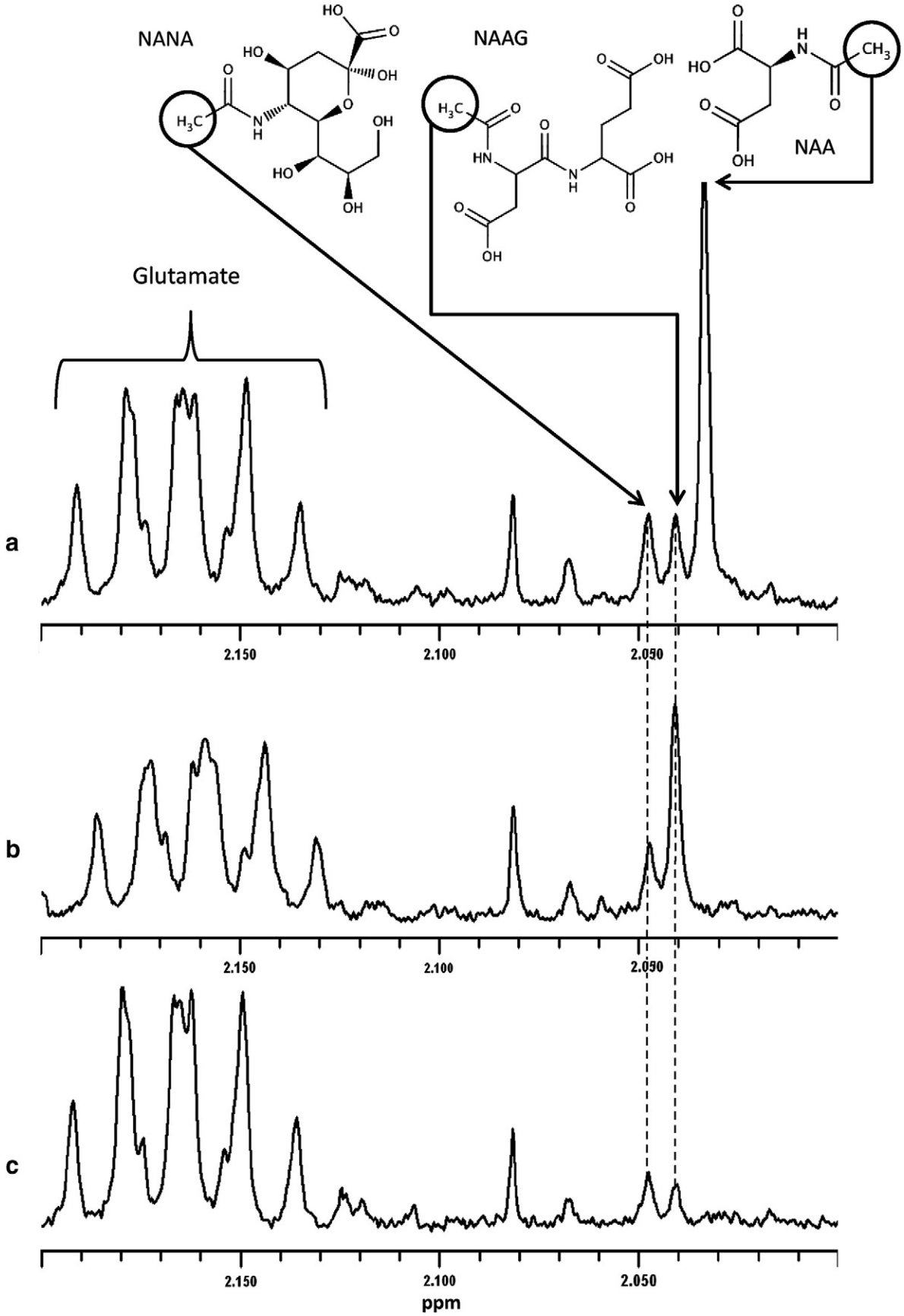

Fig. 3. [1]H-NMR spectra of CSF from 2.0 to 2.2 ppm showing the signals of NAA (singlet at 2.033 ppm), N-acetylneuraminic acid (NANA; singlet at 2.047 ppm), NAAG (singlet at 2.040 ppm) and glutamic acid (multiplet at 2.16 ppm). CSF of a patient with (a) Canavan disease showing a marked elevation of NAA (98 μmol/L) and a moderate elevation of NAAG (17 μmol/L): (b) *PLP1* duplication showing elevated NAAG (55 μmol/L), compared to (c) normal CSF spectrum.

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